REMARKS

Claims 39-43, 70 and 80 - 86 are in the case. Original claims 36-38 were canceled by amendment in favor of new claims 81-86.

All claims stand rejected variously under 35 USC § 112 and 103.

No new matter has been added.

Paragraph numbers used below correspond to those of the pending Office Action.

It is respectfully submitted that entry of the present response and amendment is proper under 37 C..F.R. 1.116 as it:

(i) places the application in condition for allowance;

(ii) does not raise any new issues requiring further search of consideration; and

(iii) places the application in better form for appeal (if necessary).

Priority

4. The Examiner notes that the claim of priority as amended in the paper received 23 December 2002 is incorrect. Applicants have amended the specification to correct the error.

Information Disclosure Statement

5. Applicants note the consideration of the IDS received on 25 November 2002.

Claim Objections

6. Claim 39 is objected to for the typographical error "elementsand" in part 2(a).

The claim has been amended accordingly

Claims Rejections – 35 USC § 112

7. Claims 42 and 43 are rejected under 35 USC § 112, second paragraph for indefiniteness. Specifically the claims depend on canceled claims. The claims have been amended to overcome this rejection.

8. Claims 82, 84 and 86 are rejected under 35 USC § 112, first paragraph for lack of enablement. Specifically, the Examiner argues that while the specification is enabling for a method for conditionally activating a transgene in a second generation plant when the promoter of the third recombinase element is not active in the common germline (using combinations of two site-directed recombination systems to cause developmentally staggered site-specific recombinations to control transgene expression), the specification is not enabling when the third recombinase promoter is active in the germline. Applicants traverse .

Claims 82, 84 and 86 have been amended to include the limitation that; P1 = a germline promoter; P2 = a floral germline promoter and P3 = a non-floral somatic tissue or germline promoter. Examples of germline promoters include 35S (non-floral) and AP3 (floral). Examples or a non-floral somatic or germline tissue prmoter includes for example the

senescene-specif promtoer (SAG) or the seed storage protein promoters such as promoters for napin, cruciferin, beta-conglycinin, and phaseolin. Under these conditions applicants submit that the activation of P3:TG will only occur in the second generation plant and not in the first generation germline cells.

Basis for the subject matter of these claims may be found in Figure 3 and on page 35 beginning at line 17 where Figure 3 is discussed.

Claim Rejections – 35 USC § 103

9. Claims 39-41, 70 and 80 remain rejected under 35 U.S.C. §103(a) as being unpatentable over Odell et al. "A" (*Mol. Gen. Genet.* 223: 369-378 (1990)), in combination with Lloyd et al. (*Mol. Gen. Genet.* 242: 653-657 (1994)), the present state of the art, and Odell et al. "B" (Use of Site-Specific Recombination Systems in Plants, in Homologous Recombination and Gene Silencing in Plants; Paszkowski, J., Ed.; Kluwer: Dordrecht, Germany, 1994; pp 219-270).

The rejection of the claims under 35 U.S.C. §103(a) is maintained for reasons of record. Specifically the Examiner argues that it would have been obvious to one skilled in the art to combine the teachings concerning single site-specific recombination systems (Odell et al. A and Lloyd et al.) with the teachings of specific promoters and transgenes (Applicant's admitted state of the prior art), and further combine these teachings with the teachings of the wide range of applications of single site-specific recombination systems (Odell et al. B) to derive the present invention. Applicants traverse.

Applicants previous arguments relating to this rejection have been considered but are not found persuasive. Specifically, applicants have argued that the art only teach instances of the single site specific recombination as opposed to the multiple use of site specific recombination of the invention. The examiner argues that the specificity of the SSR's taught in the art (Odell et al B) suggest that more than one SSR could be active and useful in a plant. Applicants submit that the level of predictability for the functioning of several independent SSR systems in one or more plants is low and one of skill in the art could not *a priori* predict with any reasonable certainty that combinations of these systems would indeed work. For example, it is well known in the art that expression of certain recombinases are toxic to some tissues resulting in poor or no expression of the recombinase. (See for example Heidmann et al, *Development Genes and Evolution* (2001), 211(8-9), 458-465; and Silver et al., *Molecular Cell* (2001), 8(1), 233-243). It was unclear at the time the invention was made what effect the expression of several different SSR elements in multiple tissues would have on plant metabolism. The expression of multiple SSR systems in multiple tissue types has not been demonstrated prior to applicants invention and one of skill in the art would not have had a reasonable expectation of success given the state of the art.

Additionally, with respect to the specific embodiment of the invention (claims 81-86), providing for the expression of a P3 driven transgene expressed only in a second generation plant, the art is silent as to how this selective expression could be achieved. For selective transgene expression in second generation plants to occur not only must the inherent unpredictability of recombinase expression be overcome but the combination of promoters must be stacked so as to produce the desired effect. This combination of promoters is clearly not suggested or anticipated in the art.

In view of the foregoing, allowance of the above-referenced application is respectfully requested.

Respectfully submitted,

LINDA AXAMETHY FLOYD, 33692
FOR
S. NEIL FELTHAM
ATTORNEY FOR APPLICANT
Registration No.: 36,506
Telephone: (302) 992-6460
Facsimile: (302) 992-5374

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TECHNICAL NOTE

Doris Heidmann · Christian F. Lehner

Reduction of Cre recombinase toxicity in proliferating *Drosophila* cells by estrogen-dependent activity regulation

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Abstract The Cre/*loxP* site-specific recombination system has been used successfully for genome manipulation in a wide range of species. However, in *Drosophila melanogaster*, a major model organism for genetic analyses, the alternative FLP/FRT system, which is less efficient at least in mammalian cells, has been established, primarily for the generation of genetic mosaics for clonal analyses. To extend genetic methodology in *D. melanogaster*, we have created transgenic lines allowing tissue-specific expression of Cre recombinase with the *UAS/GAL4* system. Surprisingly, chronic expression of Cre recombinase from these transgenes (*UAST-cre*) was found to be toxic for proliferating cells. Therefore, we also generated transgenic lines allowing the expression of Cre recombinase fused to the ligand-binding domain of the human estrogen receptor (*UASP-cre-EBD*). We demonstrate that recombination can be efficiently dissociated from toxicity by estrogen-dependent regulation of recombinase activity of the *UASP-cre-EBD* transgene products.

Keywords Cre recombinase · *loxP* · Clones · Chromosomal aberration · Toxicity

Introduction

The Cre recombinase from bacteriophage P1 has proven to be a powerful tool for manipulating pro- and eukaryotic genomes (for a review see Nagy 2000). Cre is a member of the integrase family of site-specific recombinases and catalyzes recombination between *loxP* sites. The *loxP* site is a 34-bp consensus sequence consisting of an 8-bp core spacer sequence flanked by an inverted 13-bp repeat. The demonstration that Cre recombinase is active in eukaryotic cells combined with the fact that a specific 34-bp sequence is not expected to occur by random chance within even large vertebrate genomes has encouraged the development of a large variety of genetic strategies (Nagy 2000). In particular, Cre/*loxP*-mediated conditional excision of defined sequences resulting in elimination, modification or activation of gene function in mice is now very widely used. In addition, defined chromosomal aberrations (deletions, inversions, translocations) have been created successfully, and strategies for efficient site-specific insertion of transgenes have been described.

Siegal and Hartl (1996) have recently demonstrated that the Cre/*loxP* system is applicable in *Drosophila melanogaster* as well. In this organism, however, an alternative site-specific recombination system, FLP/FRT, originally identified in yeast, was established earlier (Golic and Lindquist 1989) and is much more widely used. This FLP/FRT system has been used in *Drosophila* with great success, predominantly for the generation of genetic mosaics for clonal analyses. These experiments depend on a low recombination efficiency, just sufficient to induce recombination in a few isolated cells of a progenitor cell population followed by clonal expansion of these recombined cells. Low efficiency of recombination, however, is a severe limitation for other experimental strategies. Quantitative recombination in all cells is required for the inactivation of an allele in adult post-mitotic tissues like the *Drosophila* brain for example. In mammalian cells, the Cre/*loxP* system has proven to be far more effective than FLP/FRT (Nakano et al. 2001). The Cre/*loxP* system, therefore, might also be the system of choice for experiments requiring maximal recombination efficiency in *Drosophila*.

To expand genetic methodology in *Drosophila*, we have established transgenic lines allowing tissue-specific expression of Cre recombinase with the *UAS/GAL4* system (Brand and Perrimon 1993). Our *UAST-cre* transgene, therefore, can be combined with the existing large collection of *Drosophila* lines expressing the yeast tran-

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D. Heidmann · C.F. Lehner (✉)
Department of Genetics, University of Bayreuth, 95440 Bayreuth, Germany
e-mail: chle@uni-bayreuth.de
Tel.: +49-921-552701, Fax: +49-921-552710

scription factor Gal4 in defined tissues and at defined stages. Surprisingly, however, *UAST-cre* expression was found to be highly toxic especially in proliferating cells. To minimize these toxic effects, we have constructed additional transgenes (*UASP-cre-EBD*; EBD = estrogen-binding domain) allowing expression of Cre recombinase fused to the ligand-binding domain of the human estrogen receptor. The activity of similar fusion proteins has previously shown to be dependent on the presence of estrogen in mammalian cells (Metzger et al. 1995). Here we show that the activity of our Cre recombinase-EBD fusion proteins can also be regulated by estrogen in *Drosophila*. Thereby, activity levels can be fine-tuned allowing *loxP* site-dependent recombination without apparent toxicity.

Materials and methods

Fly stocks

The following *GAL4* lines were used: *ey-GAL4* (2/8; kindly provided by B. Dickson), *GMR-GAL4* (12; Freeman 1996), *dpp.blk1-GAL4* (40C.6; Staehling-Hampton et al. 1994), *en-GAL4*, *prd-GAL4* (Brand and Perrimon 1993), *MS1096* (Capdevila and Guerrero 1994), *F4* (Weiss et al. 1998), and *sev-hs-GAL4* (K25; kindly provided by K. Basler). *GAL4* expression in the last line is controlled by a regulatory region containing two copies of a *sevenless* enhancer and a heat-inducible promoter fragment from the *Hsp70* gene.

Transgenic lines allowing *GAL4*-dependent expression of Cre recombinase were obtained by germ line transformation with three different P element constructs. The vector pUAST (Brand and Perrimon 1993) was used for the first construct. A DNA fragment containing the complete coding sequence of Cre recombinase was amplified by polymerase chain reaction (PCR) from the plasmid pRH200 (kindly provided by Mark Siegal and Daniel Hartl, Harvard University; Mack et al. 1992) using primers (5′-GGA AGA TCT GAA TGC AAA ATG TCC AAT TTA CTG ACC-3′ and 5′-GCG GTA CCT ATC AAC TAA TTA TAG CAA TCA TTT AC-3′) introducing *Bgl*II and *Kpn*I sites at the 5′ and 3′ ends, respectively, followed by insertion into the corresponding sites of pUAST. Sequence analysis of the amplified region confirmed the presence of the correct sequence. Several independent *UAST-cre* lines were established and analyzed.

The vector pUASP (Rorth 1998) was used for the other constructs which contained fusion genes of Cre recombinase and the EBD of the human estrogen receptor. The primer EBD251 (5′-GAA GTG CGG CCG CTG AAA GGT GGG ATA CGA AAA G3′) in combination with the primer EBD3′ (5′-GTC GAC GGA TCC GAA TTC AGG-3′) was used to amplify the sequences encoding the D linker domain followed by the E ligand-binding domain of the human estrogen receptor from a cDNA plasmid (pG/ERG; kindly provided by D. Picard, University of Geneva). The resulting fragment was digested with *Not*I and *Bam*HI and inserted into the corresponding sites of pUASP. For a second construct, the ligand-binding domain without the D linker domain was amplified with the primer EBD304 (5′-CGC TCG CGG CCG CAC AGC CTG GCC TTG TCC CTG-3′) in combination with EBD3′ and inserted analogously into pUASP. In a second step the sequence encoding Cre recombinase was amplified from pRH200 using primers (5′-TCC GGT ACC CTT TAC TTA AAA CCA TTA TCT G-3′ and 5′-CGT TAG CGG CCG CTC GCC ATC TTC CAG CAG GC-3′) introducing *Kpn*I and *Not*I sites at the 5′ and 3′ end, respectively. Using these sites, the Cre recombinase fragment was introduced upstream of the estrogen receptor fragment and several independent *UASP-cre-EBD*251 and *UASP-cre-EBD*304 lines were established with the resulting constructs.

To detect Cre recombinase activity, we used the transgene ^w+^2 (Siegal and Hartl 1996). In this transgene insertion on the second chromosome, which we will designate as *lox-w-lox* in the following, a mini-*white*+ gene is flanked by *loxP* sites. Cre recombinase activity therefore results in excision of the mini-w+ gene from the chromosome.

Estrogen application

Standard fly food containing brewer's yeast, corn meal, soy meal, molasses and agar was melted in a microwave oven and cooled to about 55°C before addition of β-estradiol. Water soluble β-estradiol (E 4389, Sigma) from a 15-mg/ml stock solution was added to obtain the desired final concentration. To evaluate dose dependencies, we used final concentrations of 0.001, 0.003, 0.01, 0.03, 0.1, 0.3 and 1.0 mg/ml. For brief incubation periods, we used instant *Drosophila* food (Schlüter, Wimenden, Germany) which was dissolved in water containing β-estradiol at the desired concentration. Larvae were added to the resulting food paste on apple agar plates for the desired period. To re-isolate the larvae, food paste was scraped from the apple agar plates and mixed with 10 volumes of glucose solution (30% in H_2O). After sedimentation of food particles, the floating larvae were isolated, washed and transferred to bottles with standard fly food.

PCR assay for Cre recombinase activity

Progeny carrying *lox-w-lox*, *sev-hs-GAL4* and either *UAST-cre*, *UASP-cre-EBD*251, *UASP-cre-EBD*304, or no *UAS* transgene were collected from appropriate crosses during 24 h at 25°C. After aging for 24 h at 25°C, some of the bottles were transferred for 45 min into a 37°C water bath. After a 22-h recovery period at 25°C, larvae were isolated and genomic DNA was prepared essentially as described previously (Pirrotta et al. 1983). Genomic DNA was then used as a template for PCR with three different primers. Primer A (5′-CCT TAG CAT GTC CGT GGG GTT TGA AT-3′) was derived from a sequence present in the *lox-w-lox* transgene downstream of the 3′ *loxP* site. Primer B (5′-ATA TAT CCA TGG CAA CAC TAT TAT GCC CAC CAT TT-3′) was derived from a sequence present in the *lox-w-lox* transgene upstream of the 5′ *loxP* site. Primer C (5′-AAG TTC AAT GAT GTC CAG TGC AG-3′) was derived from a sequence present in the *lox-w-lox* transgene in between the *loxP* sites. With the combination of primers A and C, therefore, a 335-bp fragment is amplified only from the non-recombined *lox-w-lox* gene. The combination of primers A and B, in principle, allows the amplification of fragments from both the non-recombined and the recombined *lox-w-lox* transgene. However, amplification from the non-recombined *lox-w-lox* transgene is much less efficient than from the recombined *lox-w-lox* transgene because of the extensive fragment size difference (4.475 bp vs 283 bp). Amplification of the 4.475-bp fragment, therefore, was not detected and the amplification of the 283-bp fragment reflects the abundance of recombined *lox-w-lox* copies. PCR products were resolved on agarose gels and visualized with ethidium bromide. For a semi-quantitative estimation of the ratio of non-recombined and recombined *lox-w-lox* copies in the genomic DNA isolated from larvae, we performed parallel PCR experiments using standard mixtures with known amounts of the 335-bp and 283-bp products amplified with primers A and B or A and C, respectively, as template for enzymatic amplification. Moreover, in the PCR assays with genomic DNA from larvae we used template amounts resulting in comparable levels of PCR products as observed in the assays with the standard mixtures.

Detection of apoptotic cells and scanning electron microscopy

Wing imaginal discs were dissected from wandering stage third instar larvae in Ringer's solution. Vital staining of the dissected discs with 1.6 μM acridine orange was performed (Wolff 2000)

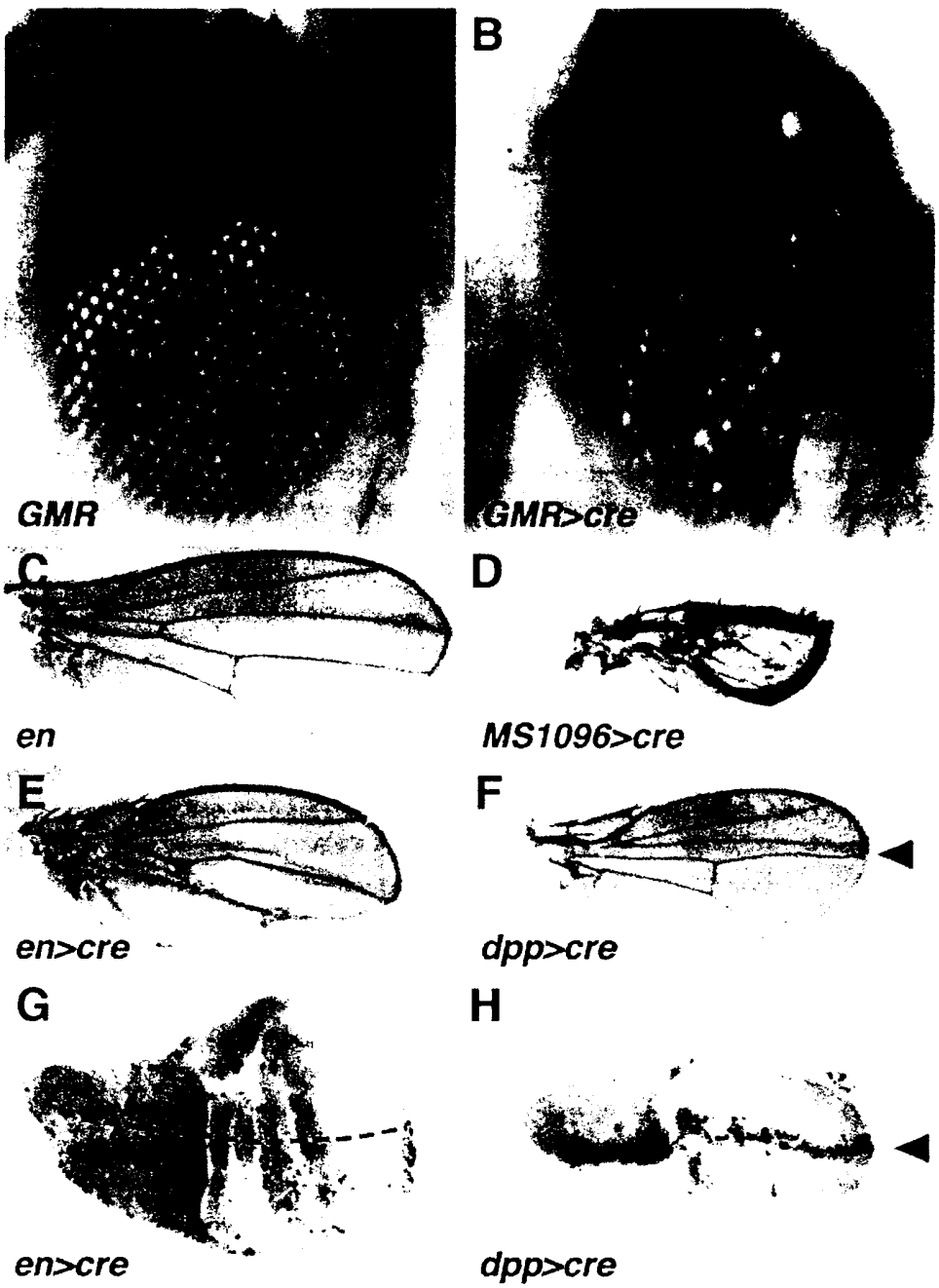

Fig. 1A–H Toxicity of Cre recombinase expression in *Drosophila*. While adult *GMR-GAL4/+* flies have wild-type eye morphology (**A**), severe abnormalities are present in eyes of *GMR-GAL4/+; UAST-cre* III.4/+ flies (**B**). Similarly, while adult *en-GAL4/+* flies have normal wings (**C**), severe truncations of the posterior wing compartment are present in *en-GAL4/+; UAST-cre* III.4/+ flies (**E**). Abnormal wing phenotypes were also observed in *MS1096/+; UAST-cre* III.4/+ (**D**) and in *dpp-GAL4/+; UAST-cre* III.4 flies (**F**) where they were restricted to the *GAL4*-expressing stripe anterior to the compartment boundary (*arrowhead*). Acridine orange staining (shown in *black*) revealed increased numbers of apoptotic cells at the third instar in imaginal discs of *en-GAL4/+; UAST-cre* II.3/+ flies (**G**) posterior to the compartment boundary (*dashed line*) or in imaginal discs of *dpp-GAL4/+; UAST-cre* II.3 flies (**H**) in a stripe anterior to the compartment boundary (*arrowhead*)

and analyzed immediately on a Leica TCS SP confocal microscope. For analysis of adult eye morphology by scanning electron microscopy (SEM) we followed standard procedures (Basler et al. 1991).

Results

To achieve tissue-specific expression of Cre recombinase with the *UAS/GAL4* system in *Drosophila*, we generated transgenic lines carrying a *UAST-cre* transgene. Unexpectedly, *UAST-cre* expression with various *GAL4* lines driving distinct temporal and spatial expression programs was found to result in severe phenotypic abnormalities. Figure 1 illustrates the abnormalities resulting from expression in eye imaginal discs (Fig. 1B, *GMR-GAL4*), or in various regions of wing imaginal discs (Fig. 1D–F, *MS1096*, *en-GAL4*, *dpp-GAL4*). Staining for apoptotic cells clearly revealed increased cell death in the *UAST-cre* expressing regions of wing imaginal discs (Fig. 1G, H). The finding that the *sev-hs-GAL4* expression resulting from a single 30 min heat shock during the first larval instar caused complete lethality in *UAST-cre*

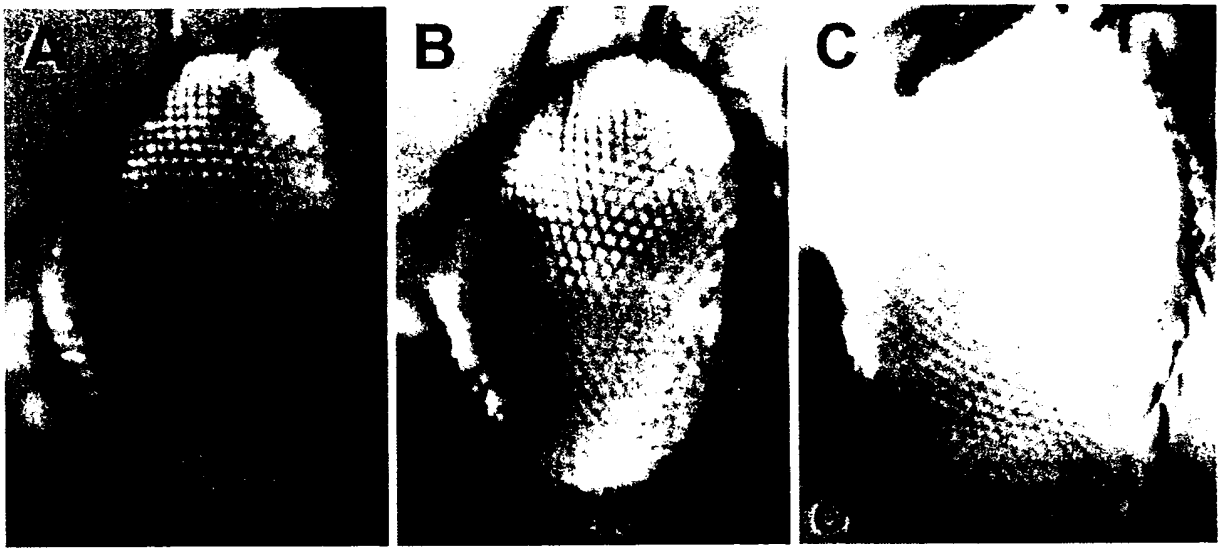

Fig. 2A–C *GAL4*-independent recombinase activity from *UAST-cre*. Eyes of flies carrying both *UAST-cre* III.4 and *lox-w-lox* (**A**) display a patchy distribution of dark and light eye color, while uniform eye color is observed in flies carrying only *lox-w-lox* (**B**) or only *UAST-cre* III.4 (**C**)

progeny further emphasized the toxicity of Cre recombinase.

Interestingly, after *UAST-cre* expression in salivary glands throughout late embryonic and larval development (with the *GAL4* enhancer trap line *F4* which results in expression levels comparable to the other *GAL4* lines used) we were unable to detect morphological abnormalities and increased apoptosis at the third instar stage. Salivary gland cells do not divide during larval development although they progress through up to ten endoreduplication cycles. In contrast, imaginal disc cells proliferate mitotically. It appears therefore that *UAST-cre* expression is especially toxic for dividing cells. Moreover, we also expressed *UAST-cre* during embryogenesis with *prd-GAL4* which directs transient expression in alternating segments during the cell division cycles 14–16 (stage 7–11). DNA labeling of embryos fixed during these and subsequent stages did not reveal differences when the extent of cell death, proliferation and differentiation in *UAST-cre* expressing and non-expressing regions was compared (data not shown). *prd-GAL4* directed *UAST-cre* expression also did not cause lethality. We conclude therefore that Cre recombinase toxicity is primarily apparent after prolonged expression in mitotically proliferating cells.

The severe toxicity resulting from prolonged *UAST-cre* expression was unexpected based on the work of Siegl and Hartl (1996). These authors have successfully established transgenes (*hsmos-cre*) which express Cre recombinase from a hybrid promoter containing elements from *Hsp70* and the mariner transposable element *Mos1*. This hybrid promoter is thought to drive constitutive ubiquitous expression. Since the *hsmos-cre* transgene does not result in phenotypic abnormalities (Siegal and Hartl 1996; and data not shown), we assumed that our *UAST-cre* transgenes result in higher expression levels. This interpretation was supported by the observation (Fig. 2) that *GAL4*-independent, basal *UAST-cre* expression resulted in a mosaic inactivation of a mini-*white*$^+$ transgene (*lox-w-lox*), in which essential sequences are flanked by *loxP* sites.

To evaluate whether toxicity results from high levels of Cre recombinase activity we generated transgenes allowing expression of a Cre protein version with inducible recombinase activity. Fusion proteins of Cre recombinase and the ligand-binding domain of the human estrogen receptor are known to display estrogen-inducible activity in mammalian cells (Metzger et al. 1995). Therefore, we generated analogous *UASP* transgenes Two different transgenes, *UASP-cre-EBD*251 and *UASP-cre-EBD*304, were constructed. The former contains a longer spacer domain between Cre recombinase and the EBD. In analogous fusions of FLP recombinase with *EBD*, the presence of the longer spacer domain has been shown to result in a higher estrogen-induced maximal recombinase activity and a higher estrogen-independent basal activity (Nichols et al. 1997).

In initial experiments, we tested whether estrogen-inducible toxicity resulted from expression of *UASP-cre-EBD*251 and *UASP-cre-EBD*304. Using *ey-GAL4* expression was restricted to eye imaginal discs. Multiple independent insertions of each transgene were analyzed and gave similar results in general, although position effects were observed as well. The majority of *UASP-cre-EBD*251 lines (16 of 26) did not result in abnormal eye morphology when larvae were raised on normal food without estrogen (Fig. 3E). In contrast, when estrogen was added, an aberrant roughened eye appearance was observed with all the tested lines (n=8; Fig. 3H). Penetrance and expressivity of this rough eye phenotype was variable with different lines. Lower concentrations of estrogen resulted in a reduced severity of the phenotypes

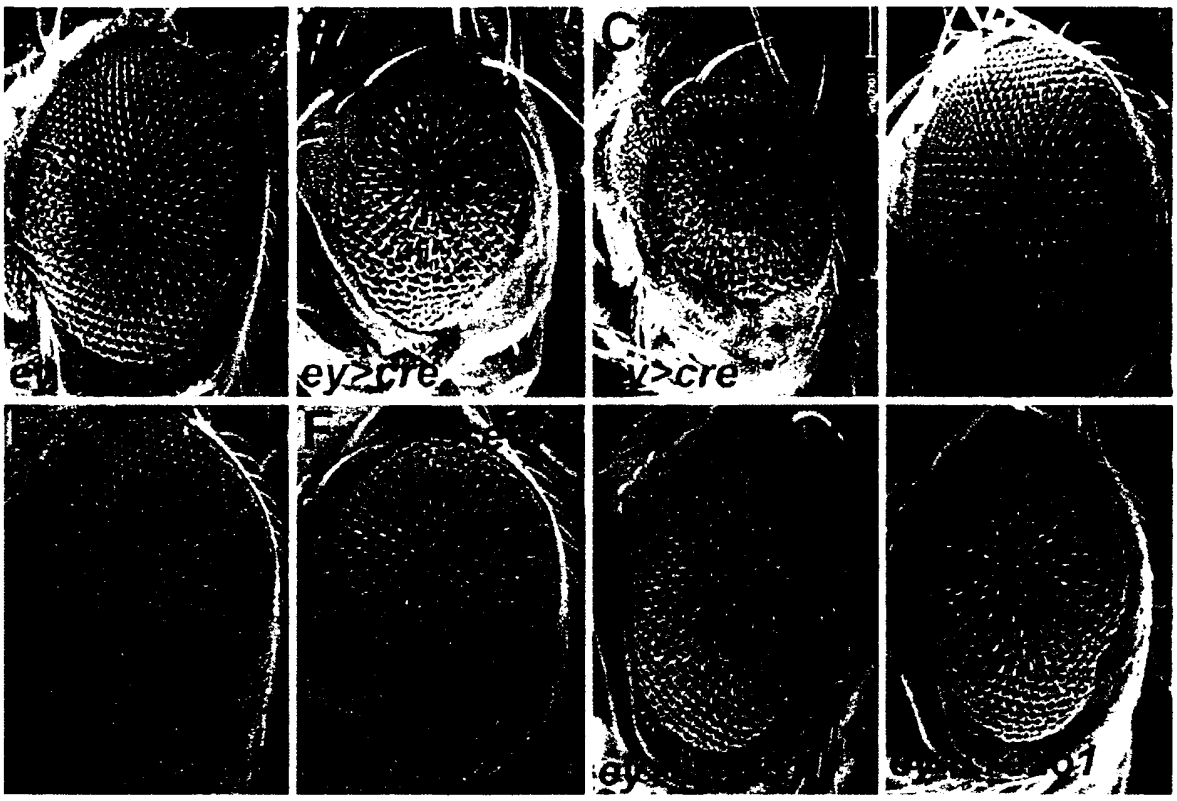

Fig. 3A–H Estrogen-dependent toxicity of *UASP-cre-EBD* expression (*EBD* estrogen-binding domain). Larvae were raised on a diet with (**A, C, D, F–H**) estrogen (+*e*) at a concentration of 0.3 mg/ml (**A. C. D. H**), 0.01 mg/ml (**F**) or 0.03 mg/ml (**G**), or without (**B. E**) estrogen (–*e*). All larvae had an *ey-GAL4* transgene and either *UAST-cre* (**B, C**), *UASP-cre-EBD*304 III.1 (**D**), or *UASP-cre-EBD*251 III.2 (**E–H**), or no *UAS* transgene (**A**). The abnormal eye morphology observed by SEM in the resulting flies reflects toxicity of Cre recombinase

Fig. 4 Estrogen-dependent recombinase activity of *UASP-cre-EBD*. DNA was isolated from *lox-w-lox* larvae carrying only *sev-hs-GAL4* (*lanes 2–5, –cre*), or *sev-hs-GAL4* in combination with either *UAST-cre* III.4 (*lanes 6–9, cre*), or *UASP-cre-EBD*304 II.2 (*lanes 10–13, cre304*), or *UASP-cre-EBD*251 III.2 (*lanes 14–17, cre251*). Before DNA isolation these larvae were raised either in the absence (-) or presence (+) of estrogen (*e*) and either not exposed (-) or exposed (+) to a heat shock (*HS*) to express *sev-hs-GAL4*. The DNA was used for PCR reactions resulting in the amplification of a 335-bp fragment from the non-recombined *lox-w-lox* allele (*lox-w-lox*) and of a 283-bp fragment from the recombined *lox-w-lox* allele (*lox*). For quantitative comparisons of the extent of *lox-w-lox* recombination we started PCR reactions in parallel using purified PCR products from non-recombined and recombined *lox-w-lox* mixed at the indicated ratios (*ratio*) as template DNA (*lanes 18–24*). *M* Molecular weight marker lane

(Fig. 3F, G). Control experiments demonstrated that estrogen has neither an effect on the wild-type eye morphology observed in flies without *cre* transgenes nor on the abnormal morphology observed in flies expressing *UAST-cre* (Fig. 3A–C). Our results demonstrate therefore that the toxicity resulting from expression of *UASP-cre-EBD*251 is estrogen-dependent.

In comparison with the *UASP-cre-EBD*251 lines, the *UASP-cre-EBD*304 lines in general resulted in a somewhat milder phenotype at a given estrogen concentration. Moreover, some *UASP-cre-EBD*304 lines did not, even at the highest estrogen concentrations, result in toxicity (Fig. 3D).

To determine whether *UASP-cre-EBD*251 and *UASP-cre-EBD*304 expressed estrogen-inducible Cre recombinase activity, we developed a semi-quantitative PCR assay

estimating the ratio of non-recombined and recombined *lox-w-lox* alleles in transgenic larvae. These larvae carried a transgene allowing heat-inducible expression of *GAL4* and *UASP-cre-EBD*251 or *UASP-cre-EBD*304 in addition to *lox-w-lox*. Larvae were raised either with or without estrogen in the food. Moreover, while one half of the larvae was kept continuously at 25°C, the other half was briefly exposed to 37°C for 45 min during the first larval instar before analysis by PCR during the second larval instar. Cre recombinase activity was found to be expressed from both *UASP-cre-EBD*251 or *UASP-cre-EBD*304 in a *GAL4*-dependent manner, and Cre recombinase activity was found to be estrogen-inducible. With *UASP-cre-EBD*304 lines (n=4) we did not observe recombinase activity in the absence of estrogen (Fig. 4, lane 12, and data not shown). In contrast, with the *UASP-cre-EBD*251 lines (n=4) we observed some estrogen-independent Cre recombinase activity (Fig. 4, lane 16, and data not shown). However, estrogen clearly stimulated recombinase activity expressed from both *UASP-cre-EBD*251 and *UASP-cre-EBD*304 (Fig. 4, lanes 13 and 17, and data not shown). This stimulation of Cre recombinase activity by estrogen appears to be dependent on the *EBD* since control experiments with *UAST-cre* did not reveal estrogen inducibility. These control experiments also confirmed that *GAL4*-independent, basal *UAST-cre* expression already results in significant Cre recombinase activity (Fig. 4, lanes 6, 7; also shown in Fig. 2).

To evaluate whether Cre recombinase activity can be adjusted to a level which is not noticeably toxic and yet allows efficient *loxP* site-specific recombination, we analyzed the eye phenotype in flies carrying *lox-w-lox* in combination with *ey-GAL4* and *UASP-cre-EBD*304 II.6. When these flies were raised on food lacking estrogen, all the adult eyes were found to have a normal morphology and a color reflecting the additive function of the three mini-w$^+$ copies present in the *lox-w-lox*, *ey-GAL4* and *UASP-cre-EBD*304 II.6 insertions (Fig. 5A). In contrast, when these flies were raised on food containing intermediate concentrations of estrogen (0.03 mg/ml), 90% of the adult eyes were predominantly of a lighter color with some darker patches (Fig. 5C), and 10% were exclusively of this lighter color (Fig. 5B) which corresponded to the eye color observed in flies carrying only *ey-GAL4* and *UASP-cre-EBD*304 II.6 but not *lox-w-lox*. Moreover, most of the eyes (76%) also had a completely normal morphology (Fig. 5B). These findings demonstrate therefore that efficient *loxP* site-specific recombination can be obtained without major toxic side effects by estrogen-dependent regulation of Cre recombinase-EBD activity.

We emphasize, however, that we were unable to obtain full penetrance of complete *lox-w-lox* recombination in the absence of morphological defects. While defects were completely absent at the lowest estrogen concentration analyzed (0.001 and 0.003 mg/ml), some eyes (6%) contained rough regions when 0.01 mg/ml was applied. The percentage of eyes with pattern abnormalities, as

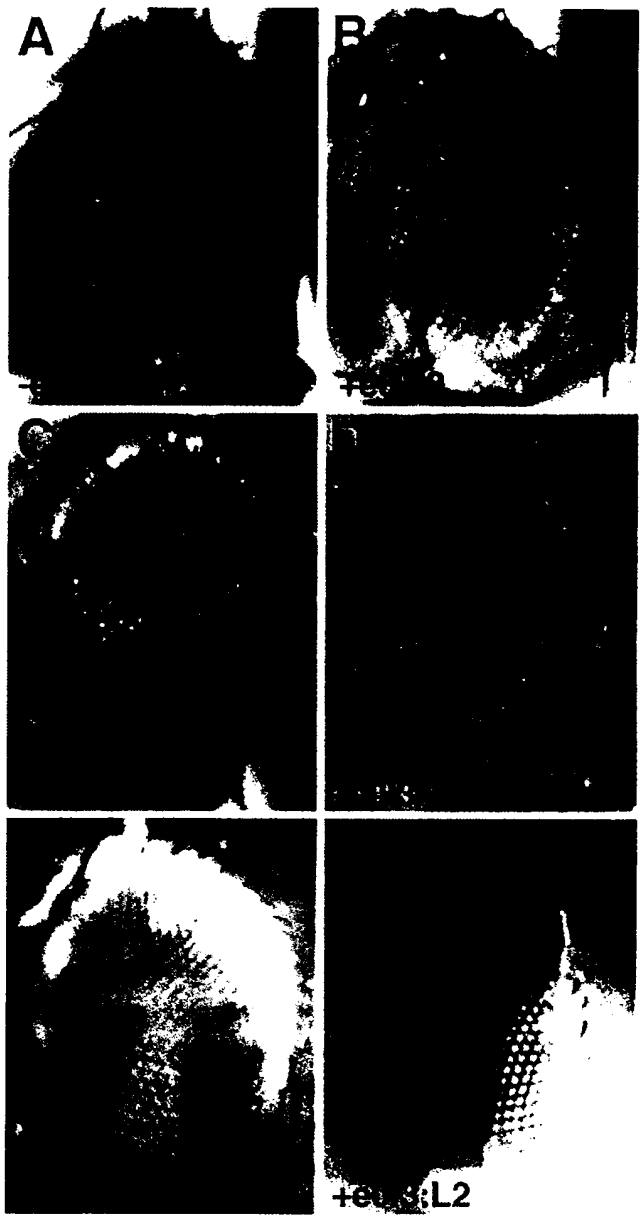

Fig. 5A–F Regulation of Cre recombinase activity and toxicity by estrogen dose and application time. Larvae with *ey-GAL4* and *UASP-cre-EBD*304 II.6 were raised in a diet without estrogen (**A**, *–e*) or with estrogen at 0.03 mg/ml (**B**, **C** *+e0.03*), or at 0.3 mg/ml (**D**, *+e0.3*) or only transiently for 3 h with estrogen during the first larval instar (**E**, *+e0.3: L1*) or during the second larval instar (**F**, *+e0.3: L2*). While *lox-w-lox* recombination does not occur without estrogen application (**A**), it can occur completely in all cells of the eye without accompanying toxicity after application of estrogen at 0.03 mg/ml (**B**). Many eyes however still contain regions with cells that have not recombined *lox-w-lox* at this concentration (**C**). Higher estrogen concentration (0.3 mg/ml) increase the efficiency of *lox-w-lox* recombination minimally but the accompanying toxicity considerably (**D**). Large continuous sectors of dark eye colors can be observed after early pulses of estrogen application during the first larval instar (**E**), while small patches of dark and light regions (see *arrow*) are observed after late pulses of estrogen application during the second larval instar (**F**)

well as the size of the affected regions within these eyes and the severity of the abnormalities, increased with higher estrogen concentrations. Interestingly, while the extent of *lox-w-lox* recombination appeared to plateau at an estrogen concentration of 0.01 mg/ml, the morphological abnormalities increased up to 0.3 mg/ml. Moreover, the morphological abnormalities often affected precisely those regions which were of a dark color (see Fig. 5D).

In an additional experiment we addressed whether Cre recombinase-*EBD* activity can be temporally controlled. In contrast to all of the previous experiments, therefore, larvae were removed from the estrogen-containing diet after a brief incubation period (3 h) and transferred to food lacking estrogen after extensive washing. As expected, small patches of lighter color were observed when the estrogen pulse was applied late during development (Fig. 5F) and very large regions of lighter color were observed after an early estrogen pulse (Fig. 5E). Importantly, the regions of darker color in the latter eyes were mostly continuous with relatively straight borders (Fig. 5E). Thus, these patches had an appearance similar to that observed for the clonal progeny of a single eye imaginal disc cell marked early during larval development. The appearance of the dark patches remaining after transient exposure of estrogen early in development was clearly distinct from the more irregular and less uniform dark patches observed after continuous exposure to estrogen. These findings indicate that Cre recombinase activity declines after removal of the larvae from the estrogen-containing food, since an extended persistence of Cre recombinase activity beyond the time of estrogen withdrawal should effectively prevent the appearance of large uninterrupted dark patches and lead to few irregular dark regions containing many smaller light clones. Moreover, the fact that Cre recombinase activity declines after removal from estrogen-containing food was further indicated by the observation that perturbation of eye morphology was no longer detected after transient feeding of estrogen at concentrations which result in obvious toxicity when present continuously (compare Fig. 5D, E).

Discussion

The Cre/*loxP* site-directed recombination system has been used for genetic manipulation in a variety of organisms. Many lines of transgenic mice expressing Cre recombinase have been described and *Drosophila* lines with a transgene thought to drive constitutive and ubiquitous expression were successfully established as well (Siegal and Hartl 1996). We were surprised, therefore, by our observation that prolonged expression of Cre recombinase during *Drosophila* development kills proliferating cells effectively. This toxicity, which appears to be dependent on high expression levels, might not be restricted to *Drosophila*. A very recent description of the consequences caused by a particular Cre transgene in mice raises the possibility that in this species Cre recombinase might also be more toxic than previously assumed (Schmidt et al. 2000). In mice, a transgene driving Cre recombinase expression in postmeiotic spermatids was found to cause abortive pregnancies with complete penetrance. A similar but reduced toxicity resulting from Cre expression in somatic cells during mouse development or in adults might perhaps have escaped detection previously because of the extensive regulative capacity of mammalian organisms and the substantial background of programmed cell death already occurring during normal development. The potential toxicity of Cre recombinase which might complicate the interpretation of some phenotypes should certainly be kept in mind.

The toxicity appears to be dependent on Cre recombinase activity. In mice, expression of an inactive Cre recombinase no longer caused male sterility (Schmidt et al. 2000). In our experiments in *Drosophila*, the expression of Cre recombinase fused to the ligand-binding domain of the human estrogen receptor was toxic only in the presence of estrogen. High levels of Cre recombinase activity therefore presumably catalyze recombination between cryptic pseudo-*loxP* sites in both the mouse and the *Drosophila* genome which result in chromosomal aberrations. This suggestion is supported by our observation that mitotically proliferating imaginal disc cells are much more sensitive than endoreduplicating salivary gland cells. Many chromosomal aberrations lead to chromosome loss during mitosis, while they have no consequences during interphase. In vitro studies have revealed a number of pseudo-*loxP* sites in yeast and mammalian genomes (Sauer 1992, 1996; Thyagarajan et al. 2000). These sites often include a number of deviations from the *loxP* consensus and yet they are capable of supporting efficient Cre-mediated recombination in vitro. Searches of the *Drosophila* genome sequence also reveal some regions that might qualify as pseudo-*loxP* sites. However, we have not analyzed the *Drosophila* genome sequence in detail because the best matches to the *loxP* consensus sequence might have been discarded as potential BAC vector sequence contamination during genome sequence assembly. Nevertheless, we emphasize that temporally limited expression of reduced Cre recombinase activity results in efficient *lox-w-lox* recombination which is not accompanied by toxicity. It is very unlikely, therefore, that the *Drosophila* genome contains pseudo-*loxP* sites that promote Cre-mediated recombination in critical regions with comparable efficiency to wild-type *loxP* sites.

As the observed toxicity is dependent on chronic expression of high levels of Cre recombinase activity it can readily be avoided. By controlling the timing and level of activity we were able to dissociate toxicity completely from *lox-w-lox* recombination. We show that inducibility of Cre recombinase activity can be achieved in *Drosophila* by expression of a fusion with an estrogen-binding domain, essentially as previously described in mammalian cells (Metzger et al. 1995).

While the activity of our Cre recombinase-*EBD* fusions could be regulated by estrogen, we were unable to

find conditions resulting in a quantitative recombination of the *lox-w-lox* allele in all of the eye imaginal disc cells in all animals. As expected, increases in estrogen concentration were found to be paralleled by a stimulation of the extent of *lox-w-low* recombination and only in the range of higher estrogen concentrations by enhanced toxicity. Concentrations resulting in a considerable fraction of progeny flies in a complete *lox-w-lox* recombination in all cells of normally patterned eyes could readily be found. Surprisingly, however, the extent of *lox-w-lox* recombination appeared to be saturated before complete penetrance and expressivity was reached. While higher estrogen concentrations further enhanced toxicity, they no longer increased penetrance and expressivity of *lox-w-lox* recombination. These results observed with our Cre recombinase-*EBD* fusions contrast with the findings of Siegal and Hartl (1996) who obtained complete penetrance and expressivity of *lox-w-lox* recombination in the absence of detectable toxicity by expressing wild-type Cre recombinase from a hybrid promoter composed of *hsp70* and *Mos1* sequences. We were readily able to confirm their results. The differences in efficiency and toxicity could reflect variations in the timing of expression. Cre recombinase is present from the onset of development when the *hsp70-Mos1* promoter is used, while we have activated the Cre-EBD fusions beginning in the first larval instar. In addition, it is conceivable that the Cre-EBD fusions have an altered, perhaps less processive, recombinase activity resulting in a higher frequency of illegitimate recombination and chromosomal aberrations. Moreover, mutations resulting within *loxP* sites during recombination attempts might also explain the difficulties in obtaining 100% of *lox-w-lox* recombination.

While the present *UASP-Cre-EBD* lines are unlikely to allow complete recombination of lox target genes, they will nevertheless be excellent tools for instance in clonal analyses. A control of Cre recombinase activity to a level appropriate for clonal analyses might also be achieved simply by using a heat-inducible promoter to regulate the level of Cre expression. However, the *UASP-Cre-EBD* lines offer the possibility to combine temporal control of recombinase activity levels and tissue-specific expression. The Cre/*loxP* system can be readily combined in *Drosophila* with the widely used FLP/FRT system, for example to generate clones within clones. This latter level of sophistication has been reached before and has revealed the highly interesting and still mysterious phenomenon of cell competition (Simpson and Morata 1981). For similar future studies, the *UAS-Cre-EBD* lines will allow for a higher efficiency and flexibility.

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Self-Excising Retroviral Vectors Encoding the Cre Recombinase Overcome Cre-Mediated Cellular Toxicity

Technique

Daniel P. Silver and David M. Livingston[1]
The Dana-Farber Cancer Institute
Harvard Medical School
Boston, Massachusetts 02115

Summary

The Cre-*lox* system is often used to manipulate sequences in mammalian genomes. We have observed that continuous expression of the Cre recombinase in cultured cells lacking exogenous lox sites caused decreased growth, cytopathic effects, and chromosomal aberrations. Cre mutants defective in DNA cleavage were not geno- or cytotoxic. A self-excising retroviral vector that incorporates a negative feedback loop to limit the duration and intensity of Cre expression avoided measurable toxicity, retained the ability to excise a target sequence flanked by lox sites, and may provide the basis of a less toxic strategy for the use of Cre or similar recombinases.

Introduction

Cre and other recombinases of the λ integrase family have opened new possibilities for the controlled manipulation of mammalian genomes. Each cleaves DNA at a specific target sequence and can ligate the newly exposed ends to the cleaved DNA at a second target sequence. Cre recombinase, the 38 kDa product of the bacteriophage P1 *cre* gene, catalyzes recombination between 34 bp target sequences termed *lox* sites (Sternberg and Hamilton, 1981). When two *lox* sites are directly repeated in *cis*, deletion of the DNA sequence between them may ensue upon expression of Cre.

The ability to catalyze these reactions efficiently in mammalian cells (Sauer and Henderson, 1988) has enabled complex genetic manipulations in animals and their cells. Cre has become the most commonly employed site-specific recombinase for higher eukaryotic genetic manipulation, particularly in mice (Kilby et al., 1993; Muller, 1999; Nagy, 2000; Rajewsky et al., 1996; Rossant and McMahon, 1999; Sauer, 1998). In this application, a segment of a gene to be inactivated or modified by Cre-mediated recombination is flanked by *lox* sites ("floxed allele"); these modifications are introduced by homologous recombination in ES cells. Being short sequences, *lox* sites generally do not perturb expression of a target gene. A mouse generated from ES cells harboring a floxed allele can be bred to a mouse transgenic for Cre. Depending on how the transgenic *cre* gene is controlled, deletion of a floxed allele occurs in a tissue and/or in a developmentally precise manner, often bypassing lethality at earlier developmental stages or avoiding other unwanted effects.

We have found that Cre can be overtly toxic to mammalian cells. This toxicity depends upon the strand cleavage activity of Cre and is, therefore, intrinsic to its activity as a recombinase. A central feature of the toxicity is genomic instability. To avoid this difficulty, we have designed a system that limits the duration and intensity of Cre expression to that necessary and sufficient for deletion of a sequence flanked on each side by lox sites. This modification eliminates the observed Cre-mediated toxicity and may have broad potential applicability.

Results

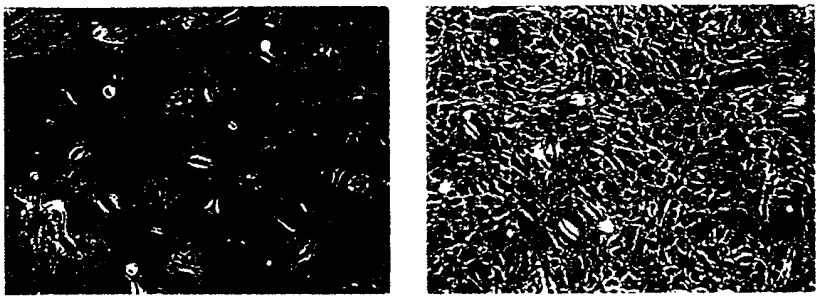
Cre Recombinase Activity Results in Cellular Toxicity

We noted that cells infected with retroviral vectors that express the Cre recombinase ceased to proliferate 5–10 days after infection. An example is shown in Figure 1A: 293xLac cells (a derivative of the human embryonic kidney cell line 293, see below) were infected with a retrovirus encoding a Cre recombinase-green fluorescent protein (GFP) fusion protein. Another plate of cells was infected in parallel with a retrovirus expressing GFP alone. The two cultures were then followed for 9 days. Cells expressing the Cre-GFP fusion protein ceased to proliferate and often spread out abnormally on the dish (Figure 1A); many syncytia were also observed. No such changes were observed in cells infected with the virus expressing GFP alone. Unmodified primary mouse embryo fibroblasts (MEFs), NIH 3T3 cells, and the human osteosarcoma cell line, U2OS, all behaved similarly, and a virus encoding Cre alone (not fused to GFP) also caused the same changes in all lines (data not shown). Thus, the toxic effect of Cre is not cell specific or GFP dependent. Furthermore, toxicity does not require the presence of exogenous *lox* sites.

A series of retroviral vectors encoding two mutant Cre proteins, R173K and Y324F, which bind to *lox* sites but cannot cleave DNA (Abremski and Hoess, 1992; Guo et al., 1999; Wierzbicki et al., 1987), were transfected as plasmids into unmodified NIH 3T3 cells (free of any introduced *lox* sites) together with pPNT, a plasmid conferring G418 resistance (Tybulewicz et al., 1991). As shown in Figure 1B, vectors expressing either wild-type (wt) Cre or the wt Cre-GFP fusion protein caused a significant decrease in the number of G418-resistant colonies compared to empty vector or vectors encoding either of the mutant Cre proteins. Furthermore, similar colony numbers were observed when the empty expression vector or any of the *cre* mutant alleles were transfected. Typical colonies from this type of experiment are shown in Figure 1C. Transient transfection of these retroviral vectors into 293T cells followed by Western analysis for Cre expression suggested that wt Cre and the two mutant proteins were equally stable, and when fused to GFP, they were expressed equivalently (data not shown). Further, Western analysis of several cell lines transiently transfected with these vectors or two commonly employed nonviral Cre expression vectors, pBS185 (Fukushige and Sauer, 1992; Sauer and Henderson, 1990) and pOG231 (O'Gorman et al., 1997; Zheng et al., 2000), showed that the retroviral-based vectors used in the present study ex-

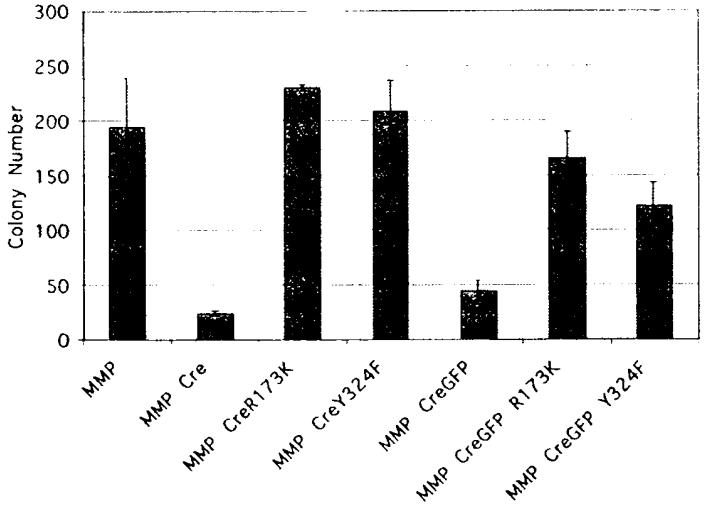
[1] Correspondence: david_livingston@dfci.harvard.edu

A



MMPCreGFP GFP

B



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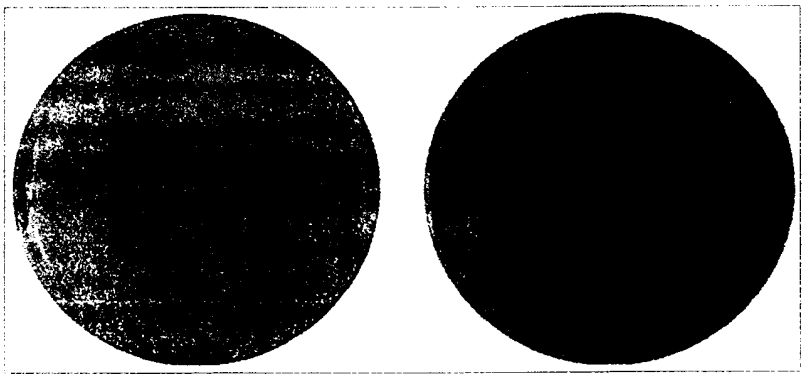

MMPCre + Neo MMPCreR173K + Neo

Figure 1. Cre Recombinase Causes Toxicity in Tissue Culture Cells

(A) 293xLac cells (see text) were infected with either MMPCreGFP (left) or a virus encoding GFP (right). The cells were passaged identically for 9 days after infection and photographed.

(B) NIH 3T3 cells (with no ectopically introduced *lox* sites) were transfected with pPNT, a plasmid encoding G418 resistance, and with plasmids encoding wt Cre, the Cre mutants, R173K or Y324F, a wt Cre-GFP fusion protein, the Cre mutants R173K or Y324F fused to GFP, or the expression vector without an insert (MMP). Cells were cultivated without selection for 2 days and then placed in G418–containing medium. Colonies were fixed, stained, and counted after 12 days of selection. The mean and standard deviation of colony counts from nine plates per expression vector are shown.

(C) Cells were treated as in (B), fixed, stained, and representative plates were photographed.

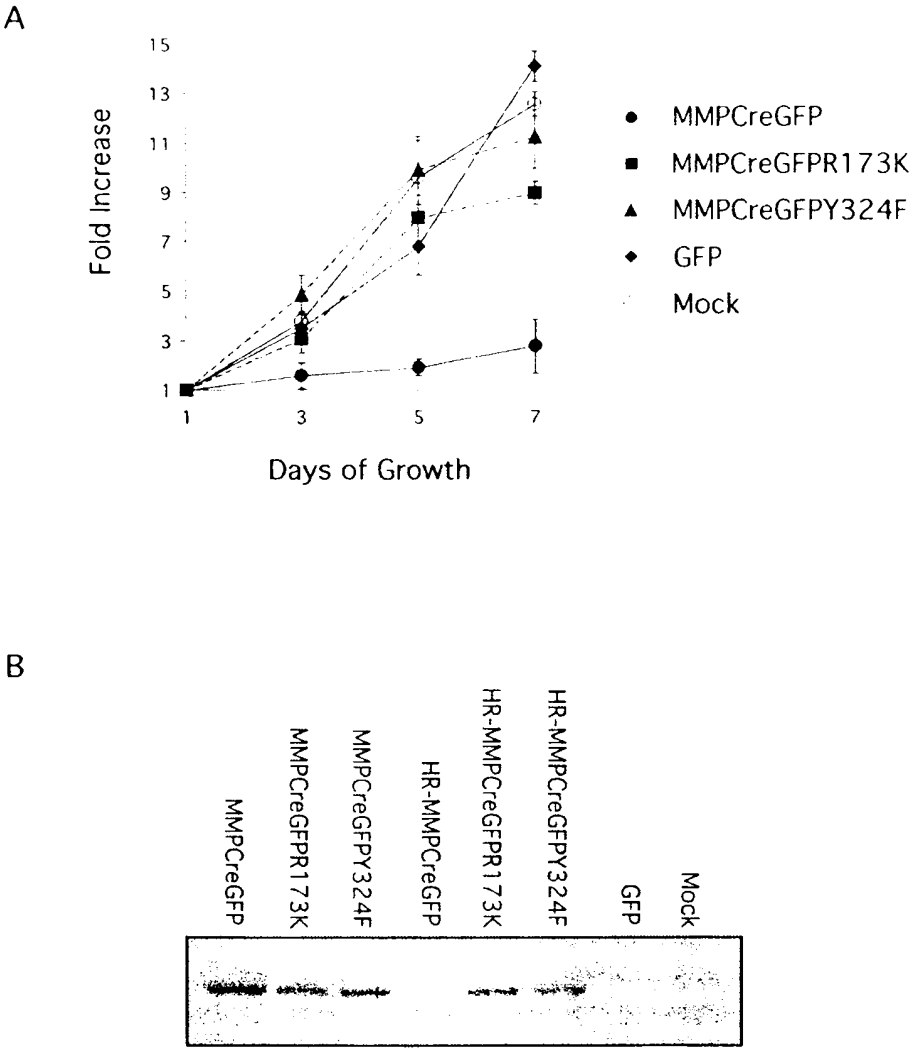

Figure 2. The Cre Recombinase Causes Growth Retardation in Mouse Embryo Fibroblasts

(A) Identical plates of early passage wt mouse embryo fibroblasts (MEFs) (with no introduced *lox* sites) were infected with the indicated retroviruses or mock infected, cultured identically for 5 days, and then seeded at the same density in multiple wells. Viable cells from three replicate wells of each infected culture were counted by trypan blue exclusion on the day after seeding and every other day thereafter. For each infection, the mean and standard deviation of cell counts, normalized to day one counts for each time point, are shown.

(B) Duplicate plates from the experiment shown in (A) and Figure 5D were used to generate cell extracts on day two of the growth curves shown. After electrophoresis of 40 ug of protein extract from each culture, the ensuing Western blots were probed with anti-Cre antibody. Lanes 1–3, 7, and 8 pertain to the growth curves shown in (A); lanes 4–6 relate to the growth curves shown in Figure 5D.

press somewhat less Cre than commonly used, nonviral vectors (data not shown). Taken together, it appears that active Cre can cause significant toxicity when introduced into a variety of cell types, that this toxicity is dependent upon the DNA cleavage function of the recombinase, and that it occurs in cells lacking ectopically introduced *lox* sites.

These findings were supported by an analysis of the proliferation of mouse embryo fibroblast after infection with retroviruses encoding Cre-GFP fusion proteins. As shown in Figure 2A, the proliferation of MEFs infected with a virus encoding wt Cre-GFP fusion protein was significantly retarded compared to mock-infected MEFs, MEFs infected with a GFP-encoding retrovirus, or MEFs synthesizing mutant Cre-GFP fusion proteins that bind *lox* sites but do not cleave them. Remarkably, MEFs infected with the wt Cre-GFP-bearing retrovirus barely proliferated and, in some experiments, underwent a decrease in cell number (Figure 2A and data not shown). There was no significant difference in the proliferation rate of mock-infected MEFs or MEFs infected with retroviruses expressing mutant Cre-GFP fusion proteins, suggesting that the toxicity observed depends upon the DNA strand cleavage activity of Cre. To check the expression levels of Cre-GFP and mutants thereof, a Western blot of total cell protein was prepared from cells on day two of the proliferation assay shown in Figure 2A and probed with anti-Cre antibody (Figure

Figure 3. Multiple Chromosomal Aberrations Are Detected in wt MEFs Infected with MMPCreGFP

Nine days after infection, metaphase chromosome spreads were prepared and stained with Giemsa. Forty-four percent of the metaphases examined revealed at least one abnormality (see table); a particularly striking metaphase is shown here, demonstrating aneuploidy, with gaps, breaks, and fragments (circled).

In the table, multiple metaphase spreads from mock-infected cells and cells infected with MMPCreGFP, MMPCreGFPR173K, and HR-MMPCreGFP were prepared as in the above panel and scored for chromosomal abnormalities. "Total number of abnormal chromosomes" refers to the number of such chromosomes in 48 metaphase spreads. The murine diploid chromosome number is 40.

)le

	Total # of Metaphases	Metaphases with Chromosomal Abnormalities(%)	Total Number of Abnormal Chromosomes	Metaphases with N>41(%)
?CreGFP	48	21 (44%)	50	18 (38%)
?CreGFPR173K	48	11 (23%)	12	5 (10%)
MMPCreGFP	48	7 (15%)	8	2 (4%)
k	48	11 (23%)	13	8 (17%)

lanes 1–3). Cre-GFP and the two mutant Cre-GFP eins were expressed at similar levels, eliminating ɔossibility that widely differing expression levels of elevant Cre-GFP alleles caused the observed differ-ɜs in cell proliferation.

› explore further the mechanism of Cre-dependent ɔity, metaphase chromosomes were prepared from MEFs 9 days after mock infection or infection with ɜes encoding wt or mutant Cre-GFP fusion proteins. ɜ infected with virus encoding wt Cre-GFP fusion ein demonstrated an increase in the percentage of ɜphase spreads showing overt chromosomal abnor-ties, including gaps, breaks, and fragments, and a ing increase in the absolute number of such abnor-ties per spread (Figure 3). Furthermore, a higher entage of metaphases obtained from cells infected wt Cre-GFP virus revealed aneuploidy than was ɜrved in mock-infected cells or in cells infected by ɜnt Cre-encoding virus. Taken together, these re-ɜ strongly suggest that prolonged exposure to a protein capable of strand scission leads, directly or ectly, to genetic instability.

lf-Excising (Hit and Run) Vector Efficiently Deletes f and a Distant Target DNA Sequence

n these results, we hypothesized that limiting the ɪsity and duration of Cre expression to the minimum level necessary for excision of a target sequence might reduce or eliminate the observed toxicity. To this end, a retrovirus that incorporates a negative feedback loop to regulate Cre expression was constructed. This regulation was accomplished by flanking the gene encoding the Cre recombinase with *lox* sites. The expectation was that upon expression, the Cre recombinase would excise the gene directing its own synthesis once the critical level of expression required for excision was reached. In assessing the properties of the self-excising Cre expression vector, the important questions were whether the Cre concentration realized in this unusual situation was sufficient to excise a target elsewhere in the genome, and, if so, whether further Cre expression terminates at that point.

The simplest embodiment of this concept in a retroviral vector would be to flank the gene encoding Cre with *lox* sites. However, because the virion RNA itself is a functional messenger, the Cre recombinase would act upon the vector DNA during the viral packaging phase of vector production and delete the Cre recombinase coding sequence. To avoid this problem, we took advantage of the fact that the U3 region of the 3′ LTR of a retroviral vector is the template for production of both U3 regions in the provirus that results after infection of a target cell (see Figure 4; among the relevant references are Gilboa et al., 1979a, 1979b; Shank et al., 1978). Be-

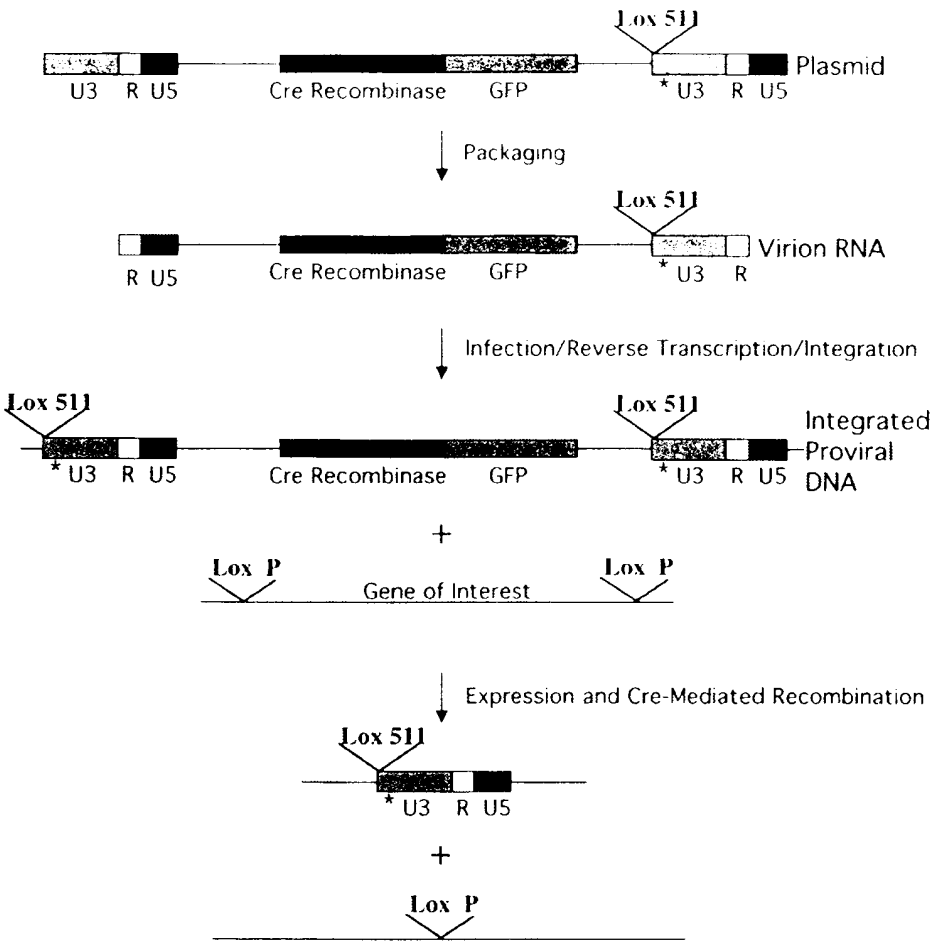

Figure 4. Mechanism of Action of HR-MMPCreGFP (Hit and Run), a Self-Excising, Cre-Encoding, Retroviral Vector

The retroviral vector contains a *lox* 511 site placed in its 3′ LTR U3 region. The structure of the expected virion RNA is shown. During the process of reverse transcription, the 3′ LTR U3 region of the viral RNA serves as a template to direct the production of the 5′ LTR U3 region. The resulting integrated provirus has the structure shown in the figure. Cre-GFP fusion protein is produced, causing deletion of its coding sequence and any other sequences elsewhere in the genome flanked by *lox* sites. The asterisk identifies the location of a StuI restriction site introduced into the *lox* 511-containing LTR to differentiate it from endogenous murine LTRs.

cause the packaging step does not entail duplication of the U3 *lox* site, this design allows packaging of the newly synthesized virion RNA without excision of the *cre* gene. After infection of a target cell, U3 *lox* site duplication ensues, permitting the development of a negative feedback loop in the target cell (Figure 4). A virus with this self-deleting characteristic can be viewed as having "hit and run" properties.

Lox sites are 34 base pair sequences consisting of two 13 base pair inverted repeats surrounding an 8 bp core (Hoess et al., 1982). The recombinase tolerates considerable sequence diversity within the core region. However, any two *lox* sites must have similar core sequences for the recombinase to utilize them efficiently in a single recombination reaction (Hoess et al., 1986). To avoid the unlikely possibility that recombination would occur between a proviral *lox* site and *lox* sites introduced elsewhere in the genome of an infected cell, *lox* 511, which differs from the widely used *lox* P site by a point mutation in the core region, was used for construction of the self-excising Cre retrovirus (Hoess et al., 1986; Lee and Saito, 1998). The design and potential action of this self-excising Cre recombinase-encoding virus, HR-MMPCreGFP (i.e., Hit and Run virus encoding Cre-GFP), is shown in Figure 4.

As an initial test of the efficiency of HR-MMPCreGFP at deleting a target sequence with flanking *lox* sites, 293xLac cells (a kind gift of Drs. R. Wells and S. Orkin) were infected in parallel with concentrated stocks of this virus and with MMPCreGFP, an identical virus except for the absence of a *lox* 511 site in its 3′ LTR. 293xLac cells are 293 cells stably transfected with a plasmid consisting of the CMV immediate early promoter followed by a *lox* P site, then a number of "stopper" features designed to prevent the expression of downstream open reading frames, then another *lox* P site followed by the β-galactosidase gene (Lakso et al., 1992). This cell line does not produce appreciable β-galactosidase until Cre-mediated recombination between the *lox* P sites removes the "stopper" sequences. Similar num-

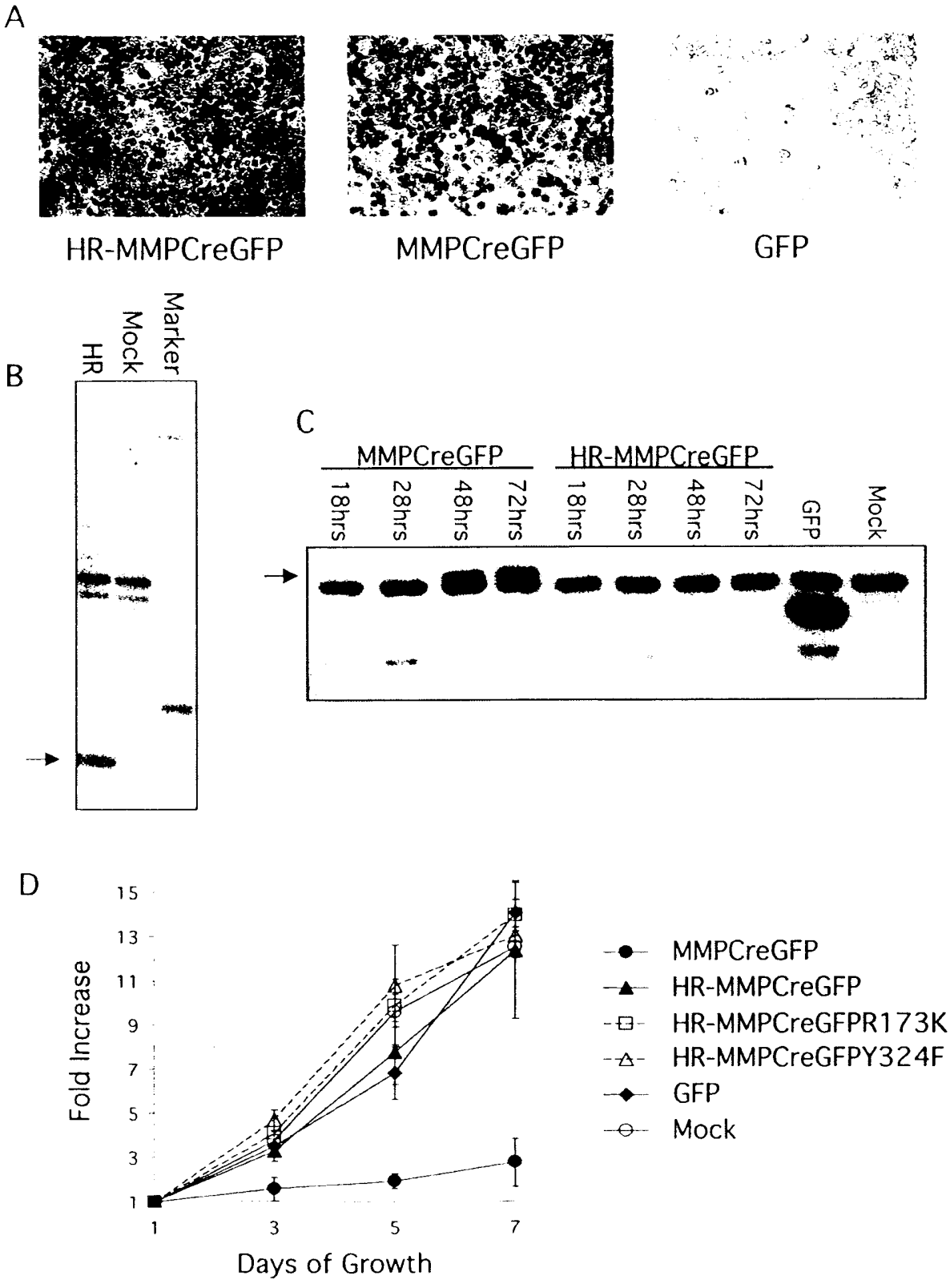

Figure 5. The HR-MMPCreGFP Virus Triggers Efficient Deletion of Both a Target Bearing Flanking *Lox* Sites and Its Own Coding Sequence without Causing Measurable Toxicity

bers of β-galactosidase-positive cells were observed 2 days after infection by the two viruses (Figure 5A, darkly stained cells). In contrast, infection with a GFP-encoding virus produced no β-galactosidase-positive cells (Figure 5A), suggesting that HR-MMPCreGFP and its non-hit and run relative are capable of causing equally efficient recombination between *lox* P sites in *trans*. Subsequent experiments using HR-MMPCreGFP to infect MEFs with *lox* sites flanking portions of BRCA1 or BRCA2 coding sequences (D.P.S., S. Ganesan, D.M.L., J. Jonkers, and A. Berns, unpublished data), MEFs with *lox* sites near the K-ras locus (D. Tuveson, D.P.S., D.M.L., and T. Jacks, unpublished data), or osteoblasts with *lox* sites flanking part of the coding sequence of Rb (D. Thomas, D.P.S., D.M.L., and P. Hinds, unpublished data) have shown that deletion of sequences between *lox* P sites can be achieved with >90% efficiency.

To investigate the details of Cre-mediated self-excision of HR-MMPCreGFP, the structure of the provirus resulting from infection was examined. Southern blotting of genomic DNA from cells prepared 6 days after infection failed to detect a *cre* gene signal after cleavage with restriction enzymes that recognize sequences located within the proviral sequences that flank the *cre* gene (data not shown). Despite the absence of a *cre* gene hybridization signal, 6 days after infection with HR-MMPCreGFP a restriction fragment of the expected size for the *lox* 511 containing LTR was detected (Figure 5B, first lane, arrow). Thus, HR-MMPCreGFP appears to infect a cell, engineer the deletion of its own *cre* sequences, and leave an LTR behind, as depicted in Figure 4. This type of Southern analysis also provides a means for determining the number of integrated proviral copies of HR-MMPCreGFP. The standard infection conditions employed here lead to an average of 1–4 proviruses per cell.

The production of the Cre recombinase protein was analyzed as a function of time after infection in Figure 5C. 293xLac cells were infected with equal amounts of concentrated supernatants of either MMPCreGFP or HR-MMPCreGFP virus, which led to the appearance of similar numbers of β-galactosidase-positive cells (Figure 5A), indicating a roughly equal ability of these stocks to promote recombination between *lox* sites. Significant quantities of Cre-GFP fusion protein were observed in cells infected with MMPCreGFP beginning 48 hr after infection (Figure 5C, arrow), but no appreciable Cre-GFP fusion protein was detected in cells infected with HR-MMPCreGFP. Thus, the amount of Cre-GFP fusion protein needed for self-excision and excision of a target elsewhere in the genome is below the limit of detection of this assay. Moreover, self-excision of HR-MMPCreGFP limits the duration and intensity of Cre recombinase expression to a level significantly below that of a typical retroviral vector without compromising the ability to excise sequences between *lox* sites.

Hit and Run Virus Does Not Cause Observable Toxicity

We have observed no phenotypic changes like those shown in Figure 1A in a variety of cell lines infected with HR-MMPCreGFP, despite prolonged culture and efficient excision of a "floxed" target sequence (data not shown). As a more specific test of toxicity, MEFs were infected with HR-MMPCreGFP or identical viruses that encode the mutant *cre* alleles described above. Cell proliferation analysis was performed in parallel with the experiment of Figure 2A; the results are shown in Figure 5D. Mock-infected MEFs and MEFs infected with HR-MMPCreGFP, HR-MMPCreGFPR173K, HR-MMPCreGFPY324F, or a GFP-only encoding virus proliferated at virtually identical rates. These results stand in contrast to what was observed with MEFs infected with MMPCreGFP, a virus identical to HR-MMPCreGFP but lacking *lox* 511 sites and, thus, unable to self-excise. A Western blot for Cre was performed on extracts obtained from cells on day two of this growth-curve experiment (Figure 2B, lanes 4–6), revealing that HR-MMPCreGFPR173K and HR-MMPCreGFPY324F express mutant Cre-GFP fusion proteins at levels similar to those in cells infected with identical viruses that lack LTR-embedded *lox* 511 sites (Figure 2B, lanes 1–3), consistent with lack of self-excision of these mutant Cre viruses, even though they contain *lox* 511 sites. Once again, because of self-excision no Cre-GFP fusion protein was detected in cells infected with HR-MMPCreGFP (Figure 2B, lane 4). Lastly, cells infected with the Hit and Run virus were analyzed for karyotypic abnormalities alongside cells infected with viruses that do not self-excise and express either wt or mutant Cre-GFP protein. There was no increase in the number of chromosomal

(A) 293xLac Cre reporter cells (see text) were infected in parallel with three viruses (10 μl each): HR-MMPCreGFP (HR indicates the presence of a *lox* 511 site in the U3 region of the 3′ LTR), MMPCreGFP (identical to HR-MMPCreGFP except for the absence of a *lox* 511 site), or a control GFP virus. Two days after infection, cells were fixed and stained for β-galactosidase activity.

(B) MEFs were infected with HR-MMPCreGFP (the Hit and Run virus), genomic DNA was isolated, and Southern blots were prepared following restriction digestion with StuI (unique to *lox* 511-bearing LTRs; see Figure 4) and KpnI (to cleave the R region of the vector LTR) and probed with a Moloney virus LTR sequence. The first lane contains DNA from MEFs infected with Hit and Run virus, the second lane contains DNA from mock-infected MEFs, and the third lane contains human DNA to which was added a plasmid encoding the Hit and Run virus to generate a marker fragment of the expected size of the *lox* 511 LTR. A band (arrow) of the size expected for the *lox* 511-containing LTR (455 nt) was detected in cells infected with the Hit and Run virus but not in mock-infected cells.

(C) 293xLac cells were infected with supernatant containing either the MMPCreGFP or HR-MMPCreGFP virus. Cell extracts were prepared at the indicated times after infection, subjected to Western blotting, and the blot was probed with anti-GFP antibody. Mock-infected 293xLac cells and cells infected with a GFP (only) virus were used as controls. A crossreacting, nonspecific band is present in all lanes and serves as a loading control; the arrow points to the band corresponding to Cre-GFP fusion protein (~66 kDa).

(D) Growth curves of additional cultures infected as part of the experiment shown in Figure 2A are shown here. N.B., the growth curves of mock-infected MEFs, MEFs infected with GFP, and MEFs infected with MMPCreGFP that were previously depicted in Figure 2A are again shown here for ease of comparison. A Western blot showing Cre-GFP fusion protein expression levels of these cell lines on day two of the growth curve is presented in Figure 2B (see lanes 4–6). Results of other experiments have shown that the same MMPCreGFP and HR-MMPCreGFP supernatants used in this experiment have equivalent ability to excise a floxed target when assayed 2 days after infection (Figure 5A and data not shown).

abnormalities or of aneuploidy in cells infected with the Hit and Run virus compared with mock-infected cells or cells infected with a mutant Cre-GFP virus (see table, Figure 3). Therefore, with respect to morphology, proliferative capacity, and chromosomal integrity, cells infected with self-excising wt Cre-GFP virus were indistinguishable from mock-infected cells, in sharp contrast to cells infected with an identical virus that cannot self-excise.

Discussion

Attempts to apply the Cre-*lox* system to cells in culture revealed that chronic Cre expression is "toxic" to a variety of tissue culture cells. Toxicity required the strand-cutting ability of the recombinase and was associated with aneuploidy and a high incidence of chromosomal aberration. Other groups have also noted aspects of Cre toxicity: Cre appears to be toxic when expressed in postmeiotic spermatids of transgenic mice (Schmidt et al., 2000), and growth-suppressive effects of Cre expression have been reported (de Alboran et al., 2001). Importantly, Anton Berns and colleagues have made observations that are both similar and complementary to those reported here regarding Cre toxicity in cultured cells (Loonstra et al., 2001). One possible explanation for these observations is that mammalian genomes contain a number of endogenous sequences that can function as targets for Cre (pseudo-*lox* sites), and continuous exposure to the enzyme is sufficient for cleavage of and, possibly, recombination among them. In the absence of mechanisms that can effectively deal with the damage associated with ongoing enzyme activity, significant genetic instability might ensue. There is considerable evidence in the literature that supports such a model. For example, Cre is capable of recognizing and catalyzing recombination at a diversity of sites related to a canonical *lox* site. Sauer has found at least ten such sites in the yeast genome (Sauer, 1992), and demonstrated Cre-dependent mitotic crossovers at one of these endogenous sites (Sauer, 1996). Moreover, Thyagarajan et al. (2000) have detected *lox*-related sites in the mouse and human genome by searching sequence databases. The Cre recombinase can cleave these sites when they are present on plasmids (Thyagarajan et al., 2000). Therefore, endogenous *lox*-like sequences that might serve as substrates for the Cre recombinase exist in the mammalian genome.

As an example of the damage that might emerge when Cre recombination occurs at selected genomic sites, recombination between ectopically introduced *lox* P sites on the same chromosome in the mouse can produce deletions, duplications, and dicentric or acentric chromosomes. These outcomes depend upon the orientation of the *lox* P sites with respect to one another and whether or not the *lox* P sites involved in a given recombination event are syntenic or are located on sister chromatids or homologs (Falco et al., 1982; Lewandoski and Martin, 1997; Ramirez-Solis et al., 1995). Similarly, intra- or interchromosomal recombination events between pseudo-*lox* sites could lead to a variety of lesions.

There are previous reports of self-deleting *cre* cassettes constructed for other purposes. Bunting et al. (1999) developed a cassette that is different from the one described here in that it expresses Cre only during spermatogenesis and also contains a ubiquitously expressed selectable marker. The intent in that study was to use the cassette first to select an ES cell that had undergone a desired homologous integration event using the ubiquitously expressed selectable marker and then to delete that same selectable marker from the germline of the mouse subsequently derived from that ES cell (Bunting et al., 1999). In addition, enhancer-deleted recombinant retroviral vectors for gene therapy applications that excise themselves have been constructed for the purpose of leaving minimal retroviral sequences integrated in a host genome (Choulika et al., 1996; Russ et al., 1996). Here, our aim was to create an efficient, general-purpose vector that would support Cre/*lox*-mediated recombination in somatic cells, including those in culture, while avoiding the aforementioned toxicity.

Since the overt abnormalities described above began to appear approximately 1 week after the introduction of Cre, it seems likely that searching for Cre toxicity soon after its expression in a cultured cell may not be revealing. Other variables may also be significant in determining the response of cells to Cre expression. The intracellular concentration of Cre may be important in determining the degree of cellular toxicity and the time when it is first detected. Furthermore, there may be significant variation among cell types in their response to Cre expression. Predicting the consequences of Cre expression in a commonly used setting, transgenic mice, is complicated by all of these factors. In addition, it may be that selection for low level and/or mosaic Cre expression occurs during the generation of certain *cre* transgenic animals, thereby avoiding lethality. Alternatively, it is conceivable that some seemingly healthy *cre* transgenic animals may manifest subtle Cre-dependent toxic phenotypes when studied closely. Likewise, Cre attack on endogenous chromosomal *lox*-like sequences or other nonspecific effects may contribute to cellular or organ-related phenotypes associated with Cre excision of a chosen target sequence. Comparative analysis of Cre-treated cells or animals that do and do not carry experimentally introduced *lox* sites but are otherwise identical would be helpful in the analysis of such phenotypes. Of special importance, the potential for mutagenesis by the Cre recombinase should be taken into account in proposals calling for its use in gene therapy protocols.

The principle of negative feedback on the production of Cre or other recombinases may have broad applicability. In the experiments described here, the level and duration of Cre expression is limited by self-excision to that minimally sufficient for excision of a simple target flanked by *lox* sites. Our data suggest that this level of expression may not be high in comparison with unrestrained expression from a retroviral LTR (see Figure 5C). Limiting expression of a given recombinase gene by such a negative feedback approach may provide a tractable scheme for reducing unwanted toxic effects while preserving the ability to recombine sequences at target signals elsewhere in the genome. For example, one could imagine producing transgenic mice, either by conventional technology or by homologous recombina-

tion in ES cells, in which the *cre* gene is flanked by *lox* sites and transcribed under the control of a tissue-specific promoter. In this setting, it may be necessary to modify Cre expression cassettes to prevent self-excision because of leaky Cre expression during cloning steps in *E. coli*; Bunting et al. have interrupted the coding sequence of Cre with an intron to solve this potential problem (Bunting et al., 1999). Conceivably, by diminishing Cre-associated toxicity, this approach would permit the construction of a wider library of *cre* transgenic mice than is currently available. Another benefit of this approach is that such animals transmitting the transgene across generations cannot express Cre at appreciable levels in the germline without compromising *cre* gene transmission. Therefore, in principle, the germline should remain free of Cre-mediated toxicity and of any genome aberrations arising from Cre activity that might otherwise be inherited.

The use of Cre to remove selectable markers and other unwanted sequences is common during the generation of knockin animals. Therefore, the germline of animals established from these ES cells has been exposed to Cre. Given this consideration, it is conceivable that at some frequency certain phenotypes of these mice may be caused in part by Cre-mediated mutations in addition to the planned knockin allele. These considerations suggest that backcrosses and analysis of multiple lines from independent ES cell clones may be useful in correlating phenotype with the presence of a given knockin allele.

The negative feedback approach has the advantage of limiting Cre expression to the level necessary and sufficient for excision. Other conditional expression strategies, such as those employing the Tet-inducible system (Gossen and Bujard, 1992; St-Onge et al., 1996), steroid receptor gene fusions (Kellendonk et al., 1996; Metzger et al., 1995; Zhang et al., 1996), or interferon regulation (Kuhn et al., 1995), do not have this self-limiting feature. A negative feedback loop could also be combined with these techniques to avoid unnecessary Cre expression while still permitting regulated induction.

Lastly, the results obtained using the Hit and Run virus for Cre-mediated recombination at *lox* sites flanking relatively short DNA segments may not be uniformly applicable to other settings where a negative feedback loop might be useful. More complicated situations, such as deletion of megabase stretches of DNA or interchromosomal recombination catalyzed by Cre, may require modification of the nature of the feedback loop to allow for higher Cre expression. For example, if the Cre recombinase coding sequence itself were located within a megabase stretch of DNA that is targeted for deletion, a negative feedback loop titrating Cre expression to the level required for this type of excision might be achieved.

Experimental Procedures

Plasmids

MMPCre was constructed using *cre* coding sequence as a template for PCR with primer 1 (GGGCACGACCATGGCCAATTTACTGACCGT ACACC) and primer 2 (GCCCGTGATCATCACTAATCGCCATCTTCC AGCAGGCG), digesting the product with NcoI and BclI, and ligating this fragment into the vector MMP (a kind gift of Drs. J.-S. Lee and R.C. Mulligan) cleaved with NcoI and BamHI.

MMPCreGFP was generated by preparing an intermediate, MMPCre3′ polylinker, which was constructed identically to MMPCre, except that primer 1 (above) and primer 3 (CGCC CTGATCAGCTATTGTCTTCCTATGCGGCCGCGGGTTTAATGGCCA AGGTGGCCCCATCGCCATCTTCCAGCAGGCG) were used for PCR. To construct MMPCreGFP, a fragment encoding GFP was prepared by digesting pEF/myc/nuc/GFP (Invitrogen) with SfiI, blunting the ends, and then digesting the product with NotI. The resulting fragment encoding GFP was ligated into MMPCre3′ polylinker prepared by cleavage with SfiI, followed by blunting of its ends and subsequent cleavage with NotI.

To generate HR-MMPCreGFP, MMPCreGFP served as a template for PCR with GTCAAGTTTGAAGGTGATACCC and GGTAGCTAGC AGGCCTATAACTTCGTATAATGTATACTATACGAAGTTATCTAGCT TGCCAAACCTACAGGTG as primers. This PCR product was cleaved with NheI and XhoI to create fragment 1. Fragment 2, containing most of the 3′ LTR, was created by cutting MMPCreGFP with EcoRI and NheI; cleaving MMPCreGFP with EcoRI and XhoI created fragment 3, which spans the 5′ LTR. Fragments 1, 2, and 3 were ligated together to create HR-MMPCreGFP. The Cre mutations, R173K and Y324F (gifts of Dr. G.D. Van Duyne), were transposed into the various wt Cre expression vectors by ligation of relevant restriction fragments bearing these mutations. PCR products and mutations were confirmed by sequencing.

Retroviral Packaging, Infection, and Transfection

Retroviral packaging was performed by transient transfection of 293T cells with packaging components and the retroviral vector using FuGene as per the manufacturer's instructions (Roche) in a variation of the procedure developed by Soneoka and colleagues (Soneoka et al., 1995). Gag-Pol expression was provided by pMD.gagpol, and envelope function was provided by pMD.G, a constitutively active version of pMDtet.G (Ory et al., 1996), to produce vesicular stomatitis virus G glycoprotein-pseudotyped retrovirus. Supernatants were harvested at 48 and 72 hr after transfection, filtered, and concentrated by ultracentrifugation using the method of Burns et al. (1993). MEFs split 1:10 or 1:12 1 day prior were infected with 40–60 μl of concentrated supernatant for 6 hr on each of 2 successive days in the presence of 8 μg/ml polybrene. All other cell lines were infected on a single day by the same protocol. Infection with HR-MMPCreGFP resulted in approximately 1–4 integrated proviruses per cell, as determined by Southern blotting (see Figure 5B). This was confirmed by infecting 293xLac cells with serial dilutions of virus followed by β-galactosidase staining. MMPCre and MMPCreGFP were used at a similar multiplicity of infection, as judged by β-galactosidase staining of 293xLac cultures infected with serially diluted virus. Western blotting with anti-Cre antibody of extracts of cells infected with MMPCre, MMPCreGFP, and the aforementioned Cre mutants (containing or lacking a *lox* 511 sequence in their 3′ LTR) confirmed that standard infection conditions for all viruses resulted in integration of ~1–4 provirus per cell (Figure 2B and data not shown).

Stable transfection of NIH 3T3 cells was performed with a 10-fold molar excess of the various Cre expression vectors to the plasmid conferring G418 resistance, pPNT (Tybulewicz et al., 1991). One microgram of pPNT was used per 10 cm plate for all transfections. Two days after transfection, cells were split into 800 μg/ml of active G418, and selection was performed over 12 days with two refeedings with G418-containing medium.

Growth Curves

Five days after initial infection, MEFs were trypsinized, quantitated by trypan blue exclusion, and plated at 7.5×10^4 cells/well of a six-well plate. The next day (day one of the growth curve) and every other day thereafter, triplicate wells were trypsinized, and viable cell content was measured by counting trypan blue-excluding cells. Results were normalized to the viable cell count on day one to determine fold increases in cell proliferation.

Southern and Western Blots

Southern blotting was performed with high-salt buffer as described (Ausebel, 1999); Hybond-XL membrane and Ambion UltraHyb were used as per the manufacturer's instructions. A probe encompassing

the LTR of MMP was prepared by digesting MMP with NheI and KpnI, isolating the resulting 451 bp fragment, and labeling with P^{32} using a Boehringer Mannheim Random Primed DNA Labeling Kit. For immunoblots, cell extracts were prepared in NETN (150 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 8.0], 0.5% NP-40).

For analysis of Cre-GFP expression, 20 μg of protein from each extract was analyzed per lane, probing with Clonetics anti-GFP antibody diluted 1:100. The secondary antibody was peroxidase-conjugated goat anti-rabbit IgG H + L (diluted 1:3000; Jackson Immunoresearch). To detect Cre itself, 40 μg of protein was analyzed, and the blot was probed with Novagen anti-Cre diluted 1:1000, with the detection reagent being peroxidase-conjugated protein A (diluted 1:2000; Amersham). Semidry Western transfers were performed as in Scully et al. (1997). Signals were detected by ECL (Amersham).

β-Galactosidase Staining and Chromosome Spreads

Cells were fixed for β-galactosidase staining in 0.5% glutaraldehyde in PBS for 5 min, washed twice with PBS, and then incubated with 2 mM $MgCl_2$, 5 mM $K_3Fe(CN)_6$, 5 mM $K_4Fe(CN)_6$, and 1 mg/ml X-gal in PBS for 3–6 hr. For chromosome spreads, cells were incubated overnight with 10 ng/ml colcemid. After detaching with trypsin, cells were then treated with 75 mM KCl for 25 min at 37°C and fixed in 3 parts methanol, 1 part acetic acid. The fixative was changed three times, and the cells were then dropped onto microscope slides and stained with Giemsa. Images were processed with the assistance of a CytoVision workstation (Applied Imaging).

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